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METHOD FOR SEQUENCING DNA USING A MICROFLUIDIC DEVICE

A method, device and reagents for the high throughput sequencing of nucleic acids.

This invention is based on a method for sequencing nucleic acids, a device for handling DNA containing samples, and a reagent kit, where the sequencing method is based on pyrosequencing.

DNA sequencing is an essential tool in basic molecular biology research. In the future it can be expected that DNA sequencing will be used in both diagnostic research as well as applied genome diagnostics.

The majority of *de novo* DNA sequencing is carried out with shot gun sequencing and with the enzymatic chain terminating method of Sanger. The sequence is generated by the resolution, using gel electrophoresis, of DNA fragments which have been prepared by elongating predetermined oligonucleotide primers. The separation of DNA fragments and the following analysis are cumbersome and great efforts have been made to automate these steps. Despite the fact that automated DNA sequencers are used in large scale genome projects there is a need for DNA sequencing devices with higher throughput, for both genome sequencing and routine clinical applications.

Pyrosequencing is a modified pyrophosphate (PPi)-based sequencing method in which PPi is detected by the release of light in the luciferase – luciferin reaction (see for example PCT patent applications WO 98/13523 and 98/28440). Each time one nucleotide molecule is incorporated into the growing DNA strand one molecule of PPi is released. The light detected is directly proportional to the number of incorporated bases in the growing DNA strand. The main drawback with this method is the number of samples that can be handled simultaneously and the speed of detection. Thus PCT application WO 98/28440 describes reactions in 96 well microtitre plates. Since the volume in each well is between 10 – 500 microlitre, the costs for the reagents are high and limit the use of the method.

When performing the pyrosequencing method in microtitre plates reaction mixes are added to the reaction chamber, but since no solution is removed from the well, the reaction can only be done a limited number of times, thereby only generating short stretches of DNA sequences. One of the major problems is to remove the excess of dNTP that can lead to misincorporation and dATP, which interferes with the light generation reaction. WO 98/28440 describes the addition of a nucleotide degrading enzyme, e.g. apyrase, to deal with this.

It is an object of the present invention to overcome several of the previous problems with pyrosequencing, such as the increasing volume when performing pyrosequencing in a microtitreplate, as well as reducing the consumption of reagents and making it feasible for analysis of several hundred samples simultaneously, thus providing a high through-put system.

Arrayed Primer EXtension (APEX), works by immobilising a large number of primers to a solid surface, thus creating a DNA-chip. These primers are constructed to be consecutively overlapping over the entire gene of interest, so that every base in the gene will have a primer to its 5'-end. By adding fluorescently labelled dideoxynucleotides, the primers will then be extended by one nucleotide using the sample DNA as template. It will thus be easy to check which nucleotide was incorporated, which in turn tells you the entire sequence of the sample DNA.

The present invention describes a method for sequencing DNA in a device with microfluidics properties and a set of reagents for its use. This microfluidic device may be in form of a disc with radially extending microchannel structures (CD form) having an inner application area that may be common for one or more microchannel structures. By spinning the disc the liquid can be driven from an applicator area into reaction and/or detection are as closer to the periphery of the disc. Liquid transportation may thus be driven by centripetal force. The microfluidic device may also have other geometrical forms.

Accordingly, in a first aspect the present invention provides a method for identifying the sequence of a portion of sample DNA, which method comprises:

(i) forming immobilised double stranded DNA on one or more reaction areas in a microchannel structure of a microfluidic device;

(ii) adding a known deoxynucleotide, (or the corresponding deoxynucleotide analogue or dideoxynucleotide) and a DNA polymerase to each of said one or more reaction areas so that extension of primer only occurs if there is a complementarity of the added deoxynucleotide or dideoxynucleotide with the strand of sample DNA that is part of the immobilised double stranded DNA;

(iii) detecting whether or not the deoxynucleotide, deoxynucleotide analogue or dideoxynucleotide added in step (ii) has been added to the primer DNA in said one or more reaction areas,

(iv) repeating steps (ii) and (iii) as required with a different deoxynucleotide (or the corresponding deoxynucleotide analogue or dideoxynucleotide).

The double stranded DNA which after step (i) is present in the reaction area consists of one strand of primer DNA and one strand of sample DNA (template). One of the strands is firmly attached to the reaction area. At least one of the strands of sample DNA and primer DNA is different for at least two reaction areas within one and the same microfluidic device.

The immobilised double stranded DNA comprising template and primer may be formed outside the microfluidic device as described in the experimental part. In the most efficient variants, it is, however, believed that the immobilised double strand is formed within the microfluidic device, for instance in the reaction chamber, by introducing separately either single or double stranded sample DNA and primer DNA. In case double stranded sample DNA is introduced in step (i) above, or in the preferred aspects described later, it has to be denatured within the microfluidic device.

The added deoxynucleotide, deoxynucleotide analogue or dideoxynucleotide in step (ii) may be labelled or unlabelled. If it is labelled the label as such is measured. Any kind of label that can be incorporated in a nucleic acid strand by the polymerase can be used, for instance a fluorescent label. If the

deoxynucleotide, deoxynucleotide analogue or dideoxynucleotide is unlabelled, nucleotide incorporation can be detected by measuring the amount of PP_i released.

The amount of single stranded sample DNA that is immobilised is typically 0.1-200 pmole but may also be as low as 1 atomole, for example 1 femtomole. The number of reaction areas may be from two upwards. Typically it is below 500,000 such as below 100,000.

In both the general and preferred aspects, the length of the elongated part of the primer may be from one base upwards. In case the method is arrayed primer extension (APEX), WO 95/00699, the elongated part of the primer is one nucleotide, for example, when using a labelled terminator e.g. dideoxynucleotide. This means that the repeating step (iv) is run at most three times.

In one aspect the present invention comprises:

(i) attaching 0.1 – 200 pmol of a primer or single stranded DNA sample to each of between one and 100,000 pre-determined areas on the surface of a microfluidic device;

(ii) hybridising small amounts, e.g. 0.1 – 200 pmol, of single stranded sample DNA or primer respectively to each of the predetermined areas;

(iii) adding a known deoxynucleotide, deoxynucleotide analogue or dideoxynucleotide and a DNA polymerase so that extension of the primer only occurs, with consequent release of pyrophosphate (PPi), if there is a complementarity with the sample DNA;

(iv) measuring the release of PPi and from which predetermined area on the device it is released;

(v) repeating steps (iii) and (iv) as required to construct a DNA sequence for the elongated primers, and hence for portions of the sample DNA.

In a preferred aspect, the present invention provides a method for identifying the sequence of a portion of sample DNA, which method comprises:

(i) adding sample DNA to a predetermined area on a microfluidic device

(ii) moving the sample to a reaction chamber on the microfluidic device

(iii) attaching the sample DNA to a surface of the reaction chamber, alternatively hybridising the sample DNA in a single stranded form to a primer attached to the reaction chamber (then to (v))

(iv) if the sample DNA has not been attached to a primer attached to the reaction chamber, hybridising a primer to the DNA in a single stranded form

(v) extending the primer in the presence of a DNA polymerase with a known deoxynucleotide (dNTP), deoxynucleotide analogue or dideoxynucleotide (ddNTP), such extension being indicated by detection of pyrophosphate (PPi) released from the extension reaction

(vi) repeating step (v) as required to establish the sequence of the extended primer.

The sample DNA to be loaded onto the microfluidic device may be an amplified sample and/or may be amplified within the microfluidic device. Amplification may involve introduction of a tag suitable for attaching the amplified DNA to a solid support.

The present invention may be applied to all fields where DNA or RNA are sequenced. These are *de novo* sequencing, resequencing of known sequences for monitoring mutation or base changes, sequencing of sequence polymorphisms and mini-sequencing where only one base is determined (including arrayed primer extension (APEX). Furthermore, the present invention may be applied to situations where the identity of a number of polymorphs is determined at the same time (see for example European Patent application 99303215.0).

The DNA to be sequenced can be of any origin: animal, plant, bacterial, or viral. This DNA can be amplified either in the device or before it is loaded onto the device.

The microfluidic device of the present invention may be analogous to those described in the literature, see patent application WO97/21090 filed by Gamera BioScience, and is preferably in the form of a disc, where the fluids are moved by

centripetal forces see for example co-pending application GB 9809943.5. The device preferably has a sample loading or application area with one or more reaction chambers and a detection chamber. Thus, a reaction can be performed in the detection chamber, and any light reaction can be detected directly when it occurs. In the case of separated chambers the flow between these can be steered by different types of barriers, like narrowed transport channels, different mechanical barriers or by surface interactions between the walls and the solution. These interactions can be of hydrophobic – hydrophilic character.

Kit of reagents:

Buffers for amplification

(vii) Enzymes for amplification, or mixed with the buffer

Sequencing buffer, luciferin

Sequencing enzymes, can also be mixed with the buffer

dCTP, dGTP, dTTP in separate buffers

dATPαS in buffer

These reagents can also be stored in a dried state e.g. glacified, direct in the disc and the reagents will then be activated first after addition of water.

Enzymes to be used in the kit:

DNA polymerase or another thermostable DNA polymerase for amplification and/or sequencing reactions e.g. Taq or other thermostable DNA polymerases

ATP sulphurylase

Luciferase

Apyrase as an optional non-preferred ingredient

Illustrative DNA polymerases are Klenow fragment polymerases. Sequenases and other 3'-5' exo- DNA polymerases, and Taq DNA polymerases and other thermostable polymerases. 3'-5' exo- DNA polymerases are preferred. Amplification reactions on sample DNA may be performed within the microfluidic device or outside it before the sample DNA is loaded onto the device.

A kit according to the invention comprises a microfabricated device, preferably in

form of a disc with radially extending microchannel structures, in combination with one, two or three of (a) Luciferase, (b) DNA polymerase, and (c) ATP sulfurylase, optionally combined with any of the above-mentioned ingredients, with preference for one or more ingredients that relate to anyone of (a)-(b) as a substrate.

The method of loading reagents and liquids to the microfluidic device can be with a dispenser, or a mechanical device for "picking" the different samples. The loading device should be able to load the different application spots onto the spinning device, preferably during the spinning of the device.

After the loading of the sample, it will be transferred by the centripetal force to the reaction chamber. In the reaction chamber the sample should be attached to a wall of the reaction chamber. If the sample is a DNA fragment, it can be attached to a surface of the reaction chamber in one of the following ways.

In the first, the DNA will be tagged at the 3'- or 5'- end during the amplification step, the tag can preferably be biotin or any other suitable tag described in the literature and suitable for attaching the tagged substance to a solid support. The surface in the reaction chamber should be activated with a substance to quickly and effectively bind to the DNA tag, preferably streptavidin will be used when the tag is biotin. The surface in the chamber can also be enlarged by the use of beads or other surface enlargement groups or structures, for example agarose or polystyrene-divinyl benzene beads (Sepharose or Source, respectively, Amersham Pharmacia Biotech AB) that are retained in the chamber, for instance by being glued to the wall of the reaction chamber. The beads or the enlargement groups may then carry the appropriate affinity group for catching the tagged DNA, for instance strepavidin in case the tag is biotin.

A second way to bind the DNA to the surface is by attaching the primer before amplification and then to perform the amplification of the sample DNA in the reaction chamber. With this approach additional coupling chemistries can be used to link the primer to the surface, such as an aminolinker on the primer with an epoxysilane treated surface.

A third way is to select the DNA sample of interest with attached primer(s). These can then be used for both attachment and act as sequencing primers, simultaneously. With this approach, the sample DNA needs to be fragmented prior to the hybridisation to the primers in the reaction chamber, following this hybridisation, the primer is extended. An advantage with this method is that several or many hundred different primers can be attached in the reaction chamber and these can be made in such a way that they cover different parts of the DNA fragment of interest, thereby the whole DNA fragment can be sequenced in one step. The distance on an unfragmented DNA molecule between sequences binding to different primers can vary between 1 to 500 bases and is most preferably 5-50 bases apart.

Binding of DNA to the reaction area may be by covalently linking one of the strands, preferably the primer, directly to the surface of a reaction area or via a specific adsorption such as via biotin-avidine as described above and other affinity pairs providing a sufficient binding to each other. A number of techniques for covalently linking DNA to solid supports are known in the scientific and patent literature.

When the sample DNA is attached to the surface it should be denatured, this can be achieved by several methods for example, hydrogen bond breaking agents, high pH or high temperature. In the present invention the preferred method is denaturing the DNA with high pH, preferably by using sodium hydroxide. Denaturation can take place either outside or inside the microfluidic device.

The following step in the invention is the elongation where DNA polymerase is added together with primer; optionally the primer can be added prior to the other compounds. The other reagents are ATP sulphurylase, luciferase, L- and D-luciferin and APS and one of the nucleotide triphosphates, dATPαS, dCTP, dGTP or dTTP. These are added sequentially, i.e. a mix with dATPαS and the other reagents, followed by a detection step and finally a wash, this is followed by dC, and then dG, and then dT or any other predetermined order. When a nucleotide is incorporated a signal is detected in the luciferase reaction and this is scored as that

base. The washing step included here solves the problem with loading the reaction mixes to one well many times and thereby getting a larger and larger volume. Since the washing here is included in the spinning device there is no need for the use of apyrase as described in Patent application WO 98/28440.

PPi can be determined by many different methods and a number of enzymatic methods have been described in the literature (Reeves *et al.*, (1969), Anal. Biochem., 28, 282-287; Guillory *et al.*, (1971), Anal. Biochem., 39, 170-180; Johnson *et al.*, (1968), Anal. Biochem., 15, 273; Cook *et al.*, (1978), Anal. Biochem., 91, 557-565; and Drake *et al.*, (1979), Anal. Biochem., 94, 117-120).

It is preferred to use luciferase and luciferin in combination to quantify the release of pyrophosphate since the amount of light generated is substantially proportional to the amount of pyrophosphate released which, in turn, is directly proportional to the amount of base incorporated. The amount of light can readily be estimated by a suitable light sensitive device such as a luminometer, or a photomultiplying device in close proximity to the device of the present invention.

Luciferin-luciferase reactions to detect the release of PPi are well known in the art. In particular, a method for continuous monitoring of PPi release based on the enzymes ATP sulphurylase and luciferase has been developed by Nyrén and Lundin (Anal. Biochem., 151, 504-509, 1985) and termed ELIDA (enzymatic Luminometric Inorganic Pyrophosphate Detection Assay). The method may however be modified, for example by the use of a more thermostable luciferase (Kaliyama *et al.*, 1994, Biosci. Biotech. Biochem., 58, 1170-1171) and/or ATP sulfurylase (Onda *et al.*, 1996, Bioscience, Biotechnology and Biochemistry, 60:10, 1740-42). This method is based on the following reactions:

$$PPi + APS \xrightarrow{\text{ATP sulphurylase}} ATP + SO^{2-}_4$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} AMP + PPi + \text{oxyluciferin} + CO_2 + h\nu$$

(APS = adenosine 5'-phosphosulphate)

The preferred detection enzymes involved in the PPi detection reaction are thus ATP sulphurylase and luciferase.

The method of the invention may be performed in two steps, as described for example in WO 93/23564 and WO 89/09283, firstly a polymerase reaction step, i.e. a primer extension step, wherein the nucleotide(s) are incorporated, followed by a second detection step, wherein the release of PPi is monitored or detected, to detect whether or not a nucleotide incorporation has taken place. Thus, after the polymerase reaction has taken place, samples from the polymerase reaction mix may be removed and analysed by the ELIDA e.g. by adding an aliquot of the sample to a reaction mixture containing ELIDA enzymes and reactants.

However, as mentioned above, the method of the invention may readily be modified to enable the sequencing (i.e. base incorporation) reactions to be continuously monitored in real time. This may simply be achieved by performing the chain extension and detection, or signal-generation, reactions substantially simultaneously by including the "detection enzymes" in the chain extension reaction mixture.

The reaction mix for the polymerase reaction may thus include at least nucleotide (deoxy- or dideoxy), polymerase, luciferin, APS, ATP sulphurylase and luciferase together with an optional nucleotide-degrading enzyme e.g. apyrase. The polymerase reaction may be initiated by addition of the polymerase or, more preferably the nucleotide. Preferably the detection enzymes are already present at the time the reaction is initiated, or they may be added with the reagent that initiates the reaction.

With the use of a microfluidic system the volumes of reagents are in the range of nanolitres compared to microlitres in the 96 well format. This will reduce the consumption of reagents a thousand fold or more.

The present invention is illustrated by the following figures, which are by way of example only, wherein:

Figure 1: A schematic drawing of the fluidic channels in the spinning device. Samples are loaded either by a mechanical device or a piezo dispenser. The reaction chamber and the detection chamber can be the same. The outlet from the chamber/s will have some barrier to stop the fluid to move during the spinning. This barrier can be a hydrophobic surface.

Figure 2: Loading a CD from a liquid train. The train is loaded from the MTP (microtiter plate) and each sample (black in the diagram) is separated with air or an inert solution, a wash solution can also be included. When the whole plate is loaded in the capillary tube, a high pressure is applied in the same or opposite flow direction and samples will then be dispensed through the piezo dispenser on to application areas of the CD surface.

Figure 3: A schematic drawing of a mechanical device for loading a spinning device. The microtitre plate at the left, a wash station in the middle and the CD where samples, reagents and liquids should be applied to. 1) start position and transfer to the CD, 2) wash the applicator means, and 3) pick up new samples. The applicator means may be in the form of pens or syringes.

Figure 4a-d: show various parts and enlargements of the microchannel structures that have been used for proof of the principle utilised in the present invention.

Figure 4a: shows the peripheral part of a circular disc. The shown part have five microchannel structure extending radially outwards.

Figure 4b: shows an enlarged view of microchannel structure K9.

Figure 4c: shows an enlarged view of the sample volume definition unit in a microchannel structure.

Figure 4d: shows an enlarged view of the reaction chamber area plus chambers for disposal of waste liquids. In particular this figure indicates variations in depth (shadowed parts I, II, III and IV).

EXPERIMENTAL

1) Materials/Investigated units

Polymerase: Klenow Fragment (3' –5'exo-) New England Biolabs (storing buffer: 10mM Tris (pH 7,4) 1mM EDTA, 1mM DTT, 50% glycerol) 5U/µl or 50U/µl: Pyrosequencing AB

Luciferase: Promega (13,33mg/ml)

Sulphurylase: Sigma (50mU/µl)

PolyvinylpyrollidonePVP: Sigma

$MgAc_2$: Merck

D-Luciferin: BioThema

DTT: Sigma

Adenosines 5′ phosphosulphate (APS): Sigma

dATPαS: Amersham Pharmacia Biotech

PPase pyrophosphatase : Sigma

dCTP, dTTP, dGTP: Amersham Pharmacia Biotech (PPi free)

Working solutions:

10 x stockA: PVP (4mg/ml); $MgAc_2$ (10.7mg/ml); D-luciferin (1.0µg/ml); DTT (1.0mM); Tris-Ac pH7.6(0.01M) APS (10mM)

Nucleotides: dATPαS(1.25mM), dCTP(0.5mM), dGTP(0.5mM), dTTP(1.25mM)

Buffers: Binding Washing Buffer (BW): 1M NaCl, 5mM Tris-HCl (pH 7.5), 0.5mM EDTA

TE Buffer: 10mM Tris-HCl, 1mM EDTA (pH 7.6)

TAE Buffer: 0,04M Tris-Acetate (7.8), mM EDTA

Particles: Source beads to which strepavidin has been attached; 15µm (APB)

Streptavidin (SA): 10mg/ml

Templates / Oligos

Template: a 50-mer oligonucleotide with known sequence tagged with biotin in the 3' end.

Primer: 24-mer oligonucleotide complementary to part of the template.

Microfluidic device material and treatment:

The microchannel structures (K7-K12) in figures 4a-d are arranged radially on a microfluidic disc. They start from a common annular inner application channel (1) and end in a common annular outer waste channel (2), coaxial with channel (1). Each inlet opening (3) of the microchannel structures may be used as an application area. Each microchannel structure is provided with a waste chamber (4) that opens into the outer waste channel (2). The flow direction is from the inlet openings (1) to the waste chamber (4). Flow is driven both by capillary action and centripetal force, i.e. by spinning the disc. Radial waste channels (5) directly connecting the annular inner channel (1) with the annular outer waste channel (2) are also shown.

Liquid passes from the inlet opening (3) via an entrance port (6) into a volume defining unit (7) and from there to a reaction chamber (10). The volume defining unit (7) has a passage into a waste channel (8) for removing excess liquid, e.g. to the annular outer waste channel (2), and a vent (9) which opens into open air. The reaction chamber (10) may become shallower (I,II,III,IV) (Fig 4d and Table) at the outlet end. A restricted channel (11) is provided between the reaction chamber (10 and the waste chamber (4). Due to the relatively large width of the waste chamber (4), there are preferably one or more supports (12) to ensure the rigidity of the chamber.

The volume defining unit (7) is U-shaped as shown in figure 4a-c with the entrance port (6) opening into the top of one of the legs of the U and the waste channel (8) starting from the other leg of the U, with a vent (9) placed at the top of this other leg. The bottom of the U-formed volume defining unit (7) is connected to the reaction chamber (10).

In addition to the application area at the inlet (3) of the structure, there may also be an additional application area (13) connected to the entrance port (6).

There is preferably also a vent (14) to open air in the reaction chamber (10). A

hydrophobic break is preferably provided at the connection (16) of the reaction chamber (10) to the volume defining unit (7).

The outer annular waste channel (2) may be sectioned so as to collect waste from a predetermined number of closely located microchannel structures.

Hydrophobic breaks were introduced by marking with an over-head pen (permanent ink) (Snowman pen, Japan): (a) between microchannel structure inlets (3) in the inner annular application channel (1), (b) each opening (15) into the outer annular waste channel (i.e. the openings of the waste chambers) and, (c) if present, also the radial waste channels (5) which connect the inner annular application channel (1) and the outer annular waste channel (2), and also the waste channel (8) which guides away excess liquid from the volume defining unit (7).

EXAMPLE 1. BEADS AS SURFACE ENLARGEMENTS AND CARRIER FOR SINGLE STRANDED DNA HYBRIDISED TO A PRIMER

Synthesis of coating agent (PEG-PEI adduct): 0.43 g of polyethylenimine (Polymin SN from BASF) was dissolved in 45 ml of 50 mM sodium borate buffer (pH 9.5) at 45°C. 5 g of glycidyl ether of monomethoxypolyethylene glycol (Mw 5 000) was added during stirring and the mixture was continuously stirred for 3 h at 45°C.

Surface treatment: A polycarbonate (**polycarbonate of bisphenol A. Macrolon DP-1265, Bayer AG, Germany)** disc as described above was placed in a plasma reactor (Plasma Science PS0500, BOC Coating Technology, USA) and treated with an oxygen plasma at 5 sccm gas flow and 500 W RF power for 10 min. After venting the reactor, the disc was immersed in a 0.1% solution of the PEG-PEI adduct in borate buffer pH 9.5 for 1 h. The disc was then rinsed with distilled water, blown dry with nitrogen and the water contact angle (sessile drop) was measured on a Ramé-Hart manual goniometer bench. The average of six equilibrium measurements (three droplets) was 24 degrees. An XPS spectrum of the treated surface gave the following molar elemental composition: 73.2%C, 3.7%N, 23.1%O, showing that the surface was essentially covered by the adsorbed

PEG-PEI adduct.

The microchannel structure was covered with a silicone rubber lid.

Streptavidin-Source 15μm particles: Source 15μm particles were oxidised with periodate, coupled with 6-aminohexanoic acid and further reacted with N-hydroxy-succinimid. Streptavidin (8mg/ml particles) was coupled to the NHS-activated particles at pH 8. Biotin capacity: 0.4 mmol/ml.

Placing SA-beads in a microchannel structure:
20 μl of a 10% Source –SA slurry were added to 0.5 μl tube and the beads washed with 1xBW. 20 μl BW buffer; 2,5μl double stranded DNA (template hybridised to the primer, (5pmol/μl) and 7.5μl TE and were added, mixed with the beads and incubated at 65°C for 10 min. The beads were then washed in TE once and TE added to a final volume of 20 μl. After each step the tube was centrifuged (30 sec.; 10.000 rpm) and the supernatant discarded.

The particles with immobilised DNA were applied as a 2% slurry to a column just before section I (about 8 nl) of the reaction chamber (10) of the microchannel structure described in **figure 4.**

Pyrosequencing reaction on the CD device

To minimise the risk for PPi contamination in the pyrosequencing mix, the test tubes used to prepare the mix were washed with 99% EtOH followed by milliQ, and dried upside down overnight.

The Pyrosequencing mix (50 μl) was prepared from the following:
33.5 μl 1xTAE
5 μl Stock A
1 μl 1xTE
4 μl Luciferase (150 ng/μl)
2.5 μl Sulphurylase (20 mU/μl)

Stepwise primer extension and detection of nucleotide insertion: Pyrosequencing mixes with nucleotides ordered in accordance with the template sequence were distributed with intermediate TAE washing to the applicator area (3). Replacement of reagents was accomplished by spinning the disc. The pyrosequencing reaction in the CD device was measured in the detector Ppy1: 1. The reaction chamber functioned as the detection chamber.

Signals were obtained which corresponded to each addition of nucleotide which could be distinguished from the background noise.

EXAMPLE 2. THE SURFACE OF THE REACTION CHAMBER AS CARRIER OF SINGLE STRANDED DNA HYBRISED TO A PRIMER

Surface treatment and immobilisation of DNA: The surface of each reaction chamber (10) was masked with Owoco Rosa (Owoco AB, Stockholm – Trangsund, Sweden). The structures were then plasma treated as described in example 1 meaning that the unmasked areas were hydrophilized. After removal of Owoco Rose, hydrophobic breaks as indicated above were made by an over-head pen (permanent ink) (Snowman, Japan). The microchannel structures were then covered with a silicone rubber lid and the channels flushed with the PEI-PEG adduct described in example 1, which adhered to the plasma treated surfaces. Thereafter strepavidin was adsorbed (3x) to the surfaces of the reaction chambers followed by a wash with TE. The reaction chamber was then filled with a solution of double stranded DNA (primer DNA hybridised to template DNA, 5pmol/µl) and incubated for 20-30 minutes to immobilise the double-stranded DNA. The channels were then washed twice with TAE.

Stepwise primer extension and detection of nucleotide insertion: See example 1.